Molecular Neurobiology (2019) 56:2440–2449
https://doi.org/10.1007/s12035-018-1229-z

The Role of Methylated Circulating Nucleic Acids as a Potential Biomarker in Alzheimer's Disease

Ming-Chyi Pai[1,2] · Yu-Min Kuo[2,3] · I-Fang Wang[4,5] · Po-Min Chiang[4] · Kuen-Jer Tsai[2,4,6]

Received: 19 April 2018 / Accepted: 8 July 2018 / Published online: 21 July 2018

Abstract

Previous studies report detection of high concentrations of circulating nucleic acids (CNAs), which are likely related to cell apoptosis, in the plasma of patients with cancers, stroke, trauma, and relapsing-remitting multiple sclerosis. However, the relationship between Alzheimer's disease (AD) and CNAs is unclear. A total of 36 adult participants (9 non-demented controls and 27 patients with AD) and patients with mild AD, who met the criteria for probable AD, were enrolled in the present study, which was conducted at the Department of Neurology of National Cheng Kung University Hospital. The CNA levels were increased in the plasma of patients with AD, culture medium of amyloid-β-treated SH-SY5Y cells, and plasma from a mouse model of AD. The CNA concentrations in the plasma were positively correlated with the cognitive scores. Further, CNAs in patients with AD contained neuronal tissue-specific methylated *LHX2*, at CpG sites 1 and 5. These results showed that the increased levels of plasma CNAs could be related to neuronal cell death that was induced by β-amyloid toxicity. Thus, the results suggested that the levels of plasma CNAs and *LHX2* methylation might serve as potential biomarkers for the diagnosis of AD, particularly during the early stages of the disease.

Keywords Alzheimer's disease · Apoptosis · Circulating nucleic acids · Methylation

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in elderly populations throughout the world. Since AD is a chronic and progressive disease, early diagnosis and intervention can have a crucial role to prevent or slow the deterioration of cognitive function in patients with AD [1, 2]. Thus, the development of sensitive diagnostic tools for the early detection of AD is important.

Circulating nucleic acids (CNAs), which are extracellular nucleic acids in peripheral plasma, serum, or lymphatic fluid, have been frequently studied and used in cancer therapy and fetal diagnosis [3]. The levels of cell-free nucleic acids are related to cell death [4]. Moreover, the CNA levels have been studied in acute illnesses, such as myocardial infarction [5], stroke [6], physical trauma [7], burns [8], and relapsing-remitting multiple sclerosis [9].

Ming-Chyi Pai, Yu-Min Kuo and I-Fang Wang contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-018-1229-z) contains supplementary material, which is available to authorized users.

✉ Kuen-Jer Tsai
kjtsai@mail.ncku.edu.tw

[1] Division of Behavioural Neurology, Department of Neurology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

[2] Alzheimer's Disease Research Centre, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

[3] Department of Cell Biology and Anatomy, College of Medicine, National Cheng Kung University, Tainan, Taiwan

[4] Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

[5] Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan

[6] Research Center of Clinical Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

Several studies, which examined variations in region- and cell-specific gene expression in the mouse [10–12] and human postmortem brain [13], demonstrated substantial brain region-specific transcription and methylation [14]. These findings supported the notion that DNA methylation is a major determinant of the functional specializations of specific regions or cell types. Intriguingly, the LIM homeobox 2 (*LHX2*) gene exhibits a higher percentage of methylation of the CpG islands, specifically in the brain, compared to the percentage in other non-neuronal tissues [15].

Since cell apoptosis increases the CNA levels [16, 17], we hypothesized that, given the atrophy of neuronal tissue in patients with AD, their levels of plasma CNAs would be increased, compared to those in healthy controls. Thus, we investigated the CNA levels and the status of brain-specific methylation of *LHX2*, as epigenetic markers, in the peripheral blood, to clarify their use as approaches for detection of early disease progression in AD.

Materials and Methods

Cell Cultures

The human neuroblastoma cell line (SH-SY5Y) was cultured as previously described [18]. Briefly, the cells were incubated in Dulbecco's modified Eagle medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10% fetal bovine serum (EMD Millipore Corporation, Billerica, MA, USA) and 1% penicillin/streptomycin and then maintained in a humidified incubator (5% CO_2, 37 °C). When the cells had grown to 80% confluence, the medium was replaced with fresh medium containing 10 μM of aggregated Aβ and incubated for 3 days. The aggregated Aβ was prepared from a solution of 10 μM of soluble $A\beta_{1-42}$ (Sigma-Aldrich Corporation, St. Louis, MO, USA) in 0.01 M phosphate-buffered saline (pH 7.4). The culture medium was collected for further study.

Animals

The animals were kept in a ventilated room under controlled conditions of 12/12-h light/dark cycle and temperature (22 ± 2 °C). The animals were given access to food and water ad libitum. This study was approved by the University Institutional Animal Care and Use Committee of National Cheng Kung University (NCKU). The experimental procedures for handling the mice were in accordance with the guidelines of the Institutional Animal Care and Use Committee of NCKU. Two different AD mouse models were used in this study. The acute Aβ-induced model was generated according to a previous protocol [18]. Briefly, the animals were anesthetized by intraperitoneal injections of 40 mg/kg of sodium pentobarbital. After the appropriate tests for residual anesthetization, each animal was placed in a stereotaxic apparatus. Microinjections of aggregated Aβ were made bilaterally into the dorsal hippocampus using a 26-gauge needle connected to a 10-μL microsyringe (Hamilton Robotics, Reno, NV, USA) at the following coordinates: 2 mm posterior to the bregma, ± 1 mm bilateral from the midline, and 1.8 mm from the surface of the skull. The total volume of the injection was 1 μL of aggregated Aβ or 1 μL of artificial cerebrospinal fluid (aCSF) at an injection rate of 1 μL/min. To allow for the diffusion of the aggregated Aβ into the surrounding tissue, the needle was left in place for an additional 5 min and then withdrawn slowly. The mice were returned to their cases for 7 days to allow for the development of the AD symptoms. The chronic AD mouse model used Tg2576 mice that were purchased from Taconic Biosciences, Inc. (Hudson, NY, USA). The Tg2576 mice contained the amyloid precursor protein double mutations of K670N/M671L that were driven by the hamster prion promoter to B6SJL F1 mice [19]. At the designated time points, peripheral blood was collected into tubes containing heparin and centrifuged at 1800×*g* for 10 min. Plasma was collected for further analysis.

AD Patients and Non-demented Controls

All studies were approved by the Institutional Review Board of NCKU Hospital, and all participants and their parents gave written informed consent. All methods were performed in accordance with the relevant laboratory/clinical guidelines and regulations. Approval was obtained to conduct a prospective study to investigate the role of CNAs in the diagnosis and prognosis of patients presenting with AD. Patients with very mild AD who met the criteria of the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association for probable AD from the Department of Neurology of NCKU Hospital were enrolled in the study [20]. The Clinical Dementia Rating (CDR) score in the patients with AD was 0.5 [21]. The cognitively intact non-demented controls with no family history of AD were randomly selected from the outpatient clinics. These subjects underwent complete neurologic and physical examinations as well as a Mini-Mental Status Examination (MMSE) and Cognitive Ability Screening Instrument (CASI) assessment. Participants with evidence of stroke, diabetes, trauma, autoimmune disorders, or known malignancy were excluded from the study. The inclusion and exclusion criteria for each group are summarized in Supplemental Table S1. Human peripheral blood was collected into tubes containing heparin on the same day as the neurologic examination, and plasma was obtained for further study.

Extraction and Quantification of CNAs

CNAs were extracted with the QIAamp Circulating Nucleic Acid kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The purified CNAs were eluted in a final volume of 50 μL of sterile water and then stored at − 20 °C. The CNAs from the culture medium or animals were quantified by semi-quantitative PCR for the β-globin gene. The primer set for CNA amplification in the culture medium included the forward primer 5′-TCCTGAGGAGAAGT CTGCCGTTA-3′ and reverse primer 5′-TCCC CAAAGGACTCAAAGAACC-3′. The amplification primers for the mouse were the forward primer 5′-GTGC ACCTGACTGATGCTGAGA-3′ and the reverse primer 5′-GGTCTCCAAAGCTATCAAAGT-3′. The data are presented as relative CNA levels normalized to the vehicle controls or wild-type mouse. The human CNAs were quantified by the real-time quantitative PCR method for the β-globin gene. The sequences of the primers used for the PCR were as follows: forward primer 5′-TGGTGCACCTGACTGATGCT-3′, reverse primer 5′-ACTTCATCGGCGTTCACCTTT-3′, and the FAM dye-labeled minor groove binder probe 5′-AGAA GGCTGCTGTCTC-3′. The fluorogenic PCRs were performed using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific Inc.). The calibration curve of each plate was calculated based on a dilution series of the plasmid that carried the insertion of the PCR product from the real-time PCR system for the human β-globin gene at 9×10^6, 9×10^4, 4.5×10^2, 225, 56.25, 28.12, and 14.06 copies. Standard blank samples of water were used as the negative control, and the patient samples were amplified in duplicate and analyzed in three independent experiments. The quantitative results were expressed as kilogenome-equivalents per liter, as described previously [22]. One genome-equivalent was defined as the amount of a target sequence contained in a single diploid human cell.

Cognitive Function Tests

The scores on the MMSE range from 0 (severe impairment) to 30 (no impairment) [23]. A MMSE score above 27 is considered to reflect normal cognition [20]. The CASI has frequently been used as a dementia screening tool. The current scoring system for the CASI involves summing the scores from each of the nine domains to obtain a total score ($CASI_T$), which ranges from 0 to 100 [24]. Higher scores indicate better cognitive ability. If the subtracted cutoff scores from the $CASI_T$ score (CASIT-cutoff) were more than zero, cognitive function was identified as normal. An earlier study has demonstrated that assessments of the combined short-term memory and orientation domain scores ($CASI_R$) in the CASI test were more effective than using $CASI_T$ for dementia screening [25].

Pyrosequencing

To analyze the methylation of *LHX2*, we used the quantitative methylation analysis to determine DNA amounts after the whole bisulfitome amplification method [26]. Human CNAs were modified by treatment with sodium bisulfite according to the manufacturer's instructions for the bisulfite conversion kit (QIAGEN GmbH). The converted samples were stored at − 20 °C. Genome-wide amplification of the plasma CNAs was performed using the Whole Bisulfitome Kit (QIAGEN GmbH). To quantify the percentage of methylated cytosine in individual CpG sites, bisulfite-converted CNAs that were subjected to genome-wide amplification were pyrosequenced using a pyrosequencing system (QIAGEN GmbH) according to the manufacturer's standard protocol. The PCR primers were 5′-AGTT TTTTTTTTATGGTTTTTATAGG-3′ (forward) and the 3′-biotinylated reverse primer 5′-CAATAAACCAAACT ATCCTTCAT-3′. The PCR products then underwent pyrosequencing analyses. The primer used for pyrosequencing was 5′-GTTGTTATTTGGGTATTT-3′, which was designed to probe a series of five CpG dinucleotides in *LHX2*. The methylation values at single CpG positions were assessed using PyroMark Q24 1.0.10 software.

ImageJ

The optical density of the β-globin bands was quantified using ImageJ software (https://imagej.nih.gov/ij/). The protocol can be found at http://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf.

Statistics

The data are expressed as mean ± standard error of the mean. The statistical analysis was performed using SPSS software (IBM Corporation, Armonk, NY, USA) or Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) to identify significance ($p < 0.05$). Non-normally distributed data were analyzed using the Mann-Whitney *U* or Kruskal-Wallis non-parametric tests. Correlation analyses between the plasma CNAs and variables were performed using Spearman's rank correlation coefficients.

Data Availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files or are available from the corresponding author on reasonable request.

Results

Aggregated $A\beta_{42}$ Increased the Levels of CNA in SH-SY5Y Cells and Tg2576 Mice

Higher levels of CNA are thought to result from their increased release after cell apoptosis. Thus, we examined the toxic effects of amyloid-β (Aβ) on cell death and CNAs production in vitro. We incubated SH-SY5Y cells with 10 μM of aggregated Aβ for 72 h to induce cell death (Fig. 1a). CNA levels were measured in the culture medium using semi-quantitative polymerase chain reaction (PCR). Compared with the vehicle control treatment, CNA levels were significantly increased after aggregated Aβ treatment (Fig. 1b; $*p < 0.05$).

To examine the effects of aggregated Aβ on plasma CNAs, we examined the levels of CNA in the following two mouse models of AD: an acute AD mouse model and Tg2576 mice, to monitor AD-like feature development and AD disease progression, respectively. The levels of plasma CNAs were significantly increased in the Aβ-injected mice compared with artificial cerebrospinal fluid (aCSF)-injected mice in the acute AD mouse model (Fig. 1c; $*p < 0.05$). To further confirm that CNA level accompanies AD pathogenesis, we measured the CNA levels during disease progression in Tg2576 mice. We collected mouse plasma from the facial vein of mice of different ages. We noted that the CNA levels increased progressively, peaking at 6–8 months (Fig. 1d; $*p < 0.05$, $**p < 0.01$). This period coincided with the onset of memory decline and was prior to the formation of Aβ plaques, which occurred at 12 months. These results suggested that the levels of plasma CNAs were increased by the neurotoxicity of Aβ, which indicated that Aβ has cytotoxic effects, even during the early loss of neuronal function.

Patients with AD

Previous studies demonstrated that the levels of plasma CNAs are high in patients with various diseases [27, 28]. Thus, we excluded all patients with comorbidities (Supplemental Table S1) to limit any confounding effects of other diseases. The clinical characteristics of the 36 adult participants who were enrolled in our study are shown in Supplemental Table S2. Cognitive function was tested using the Mini-Mental State Examination (MMSE) or Cognitive Ability Screening Instrument (CASI) total ($CASI_T$). The scores of the patients with AD were lower than those of the controls (Supplemental Table S2; $***p < 0.0001$). Since CASI scores were significantly associated with an individual's level of education, the participants were stratified with different cutoff scores, according to their educational level. The cutoff scores that were subtracted from the $CASI_T$ score ($CASI_T$-cutoff) indicated that patients with AD in the present study had cognitive dysfunction.

CNAs in Patients with AD

To precisely quantify the levels of plasma CNAs in patients with AD, we developed a real-time quantitative PCR protocol for detection of β-globin CNAs with size about 70 bp (Supplemental Fig. S1). The CNA concentrations in the control group were significantly lower than those in the patient group (Fig. 2a; 1852 ± 861.7 vs. 41,831 ± 25,204 kilogenome-equivalents/L, $**p < 0.01$). These results suggested that the plasma CNA concentrations were significantly increased in patients with AD compared with non-demented controls. To further examine if the concentrations of plasma CNAs could act as a diagnostic predictor, a receiver-operating characteristic (ROC) curve analysis was performed to assess the diagnostic accuracy of AD at different plasma CNA concentrations (Fig. 2b). The area under the curve (AUC) was 0.807 (excellent discrimination), and the 95% confidence interval (CI) was 0.657–0.956. At a plasma CNA concentration cutoff of 4, 161.31 kilogenome-equivalents/L, the optimal sensitivity and specificity were 67% (95% CI, 46.04–83.48%) and 89% (51.75–99.72%), respectively. Moreover, we divided the participants into two groups of higher and lower concentrations of plasma CNAs (optimal cutoff = 161.31 kilogenome-equivalents/L), and calculated the odds ratios (Supplemental Tables S3 and S4). The odds ratio for AD was 16, and the ratio of cognitive impairment was 8. These results indicated that higher concentrations of plasma CNAs were strongly associated with AD or cognitive impairment.

The CNA concentrations of all of the participants and those in the AD group did not correlate with any clinical parameters (Supplemental Tables S5 and S6) or cognitive function test scores (Fig. 3a). Cognition is defined as being free of dysfunction when the $CASI_T$-cutoff score is higher than zero. Therefore, we compared the concentrations of plasma CNAs in patients with AD and normal cognition ($CASI_T$-cutoff score > 0; Table 1) with those of the control participants. Patients with AD and normal cognition had higher concentrations of plasma CNAs (Fig. 3b; $*p < 0.05$). We confirmed this correlation with the CASI-combined short-term memory and orientation domain scores ($CASI_R$); we demonstrated that the concentrations of plasma CNAs in patients with AD and normal cognition ($CASI_R > 22.5$; Table 1) were greater than those of control participants (Fig. 3c; $*p < 0.05$). Patients with AD were further classified according to dementia severity (mild or moderate to severe), with a cutoff score of 10 [29]. Intriguingly, the levels of plasma CNAs increased gradually according to dementia severity and peaked in patients who were classified as having mild or moderate cognitive impairment (Fig. 3d; $*p < 0.05$). Further, we confirmed the correlation between plasma CNA concentrations and dementia severity. The patients were classified according to their MMSE score as having normal

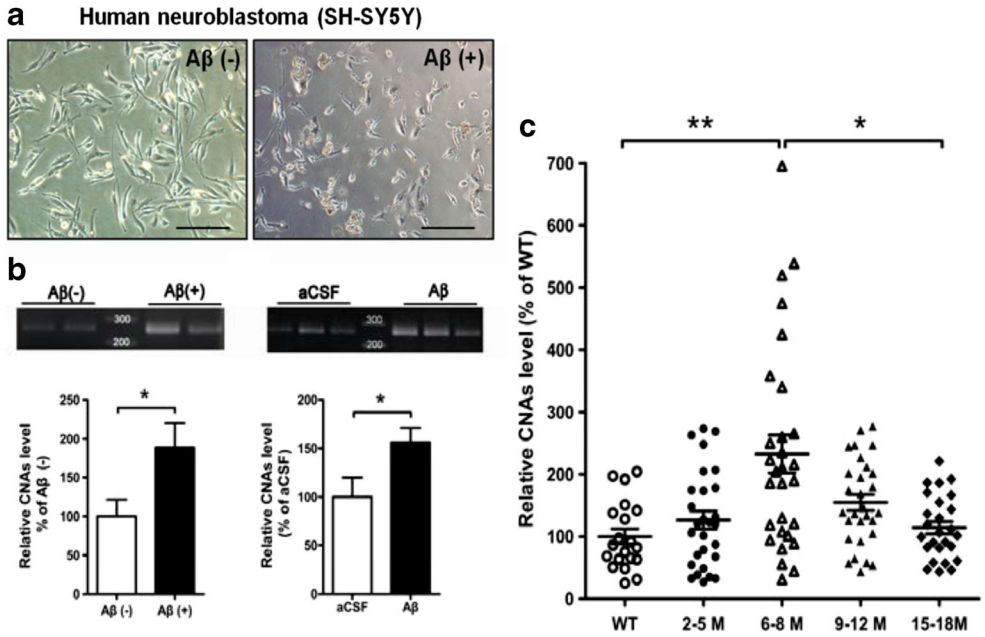


Fig. 1 Aggregated $A\beta_{42}$ increases the amount of circulating nucleic acids (CNAs) released in vitro and in vivo. **a** The photographs show SH-SY5Y cells that were treated with vehicle or aggregated Aβ. Note that Aβ that was aggregated for 72 h induced the cell death of the SH-SY5Y cells. Scale bar = 100 μm. **b** The CNAs were purified from the culture medium of the SH-SY5Y cells and analyzed for β-globin gene. Note that the CNA levels in the SH-SY5Y cells were significantly increased after treatment with aggregated Aβ compared with treatment with vehicle control. The data are expressed as the mean ± standard error of the mean (SEM). $n = 4$ in Aβ (−) and $n = 6$ in Aβ (+). Mann-Whitney U test, $^*p < 0.05$. **c** The CNAs were extracted from the plasma of the mice that were injected with artificial cerebrospinal fluid (aCSF) or aggregated Aβ. Note that the levels of the CNAs were higher in the Aβ-injected mice than in the aCSF-injected mice. The data are expressed as the mean ± SEM. $n = 8$. Mann-Whitney U test, $^*p < 0.05$. **d** CNAs were extracted from the plasma of Tg2576 mice and analyzed through semi-quantitative PCR. Note that the CNA levels increased and peaked at 6–8 months. The data are expressed as the mean ± SEM. $n = 15$ in wild type (WT), $n = 19$ in 2–5 months (M), $n = 26$ in 6–8 M, $n = 25$ in 9–12 M, and $n = 18$ in 15–18 M. The relative amount of CNAs at the indicated age was normalized with that of the wild-type mice. $^*p < 0.05$; $^{**}p < 0.01$ by Kruskal-Wallis test

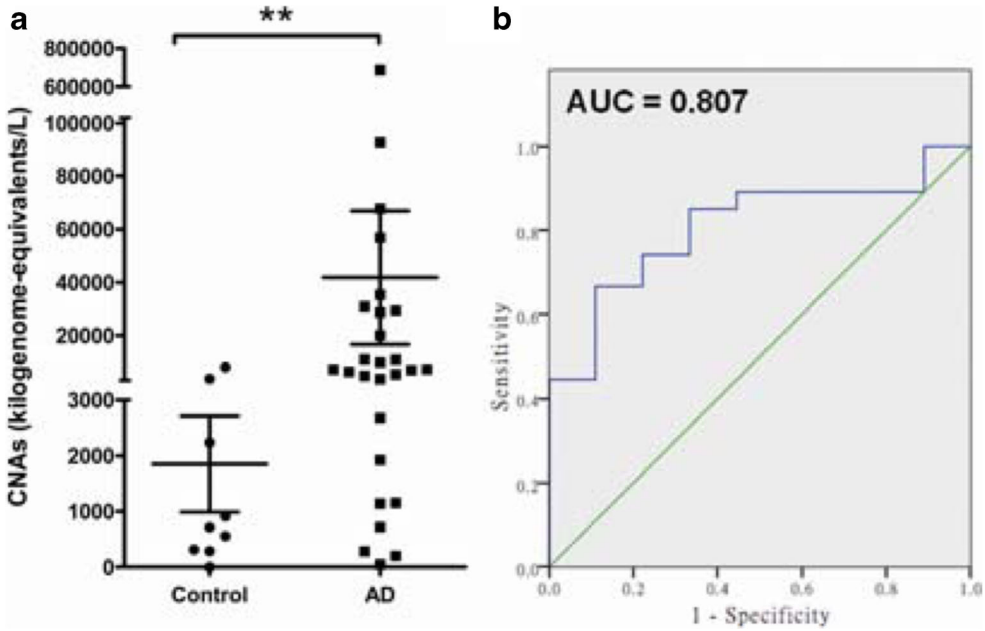


Fig. 2 Plasma CNA levels were increased in the patients with Alzheimer's disease (AD). **a** The CNA levels were quantified using real-time absolute quantitative PCR. The quantitative results are shown as kilogenome-equivalents per liter. One genome-equivalent per liter was defined as the amount of a target sequence contained in a single diploid human cell per 1 L of plasma. The data are expressed as mean ± SEM. The sample sizes were 9 in the control group and 27 in the AD group. The CNA levels were significantly increased in the AD patients compared with the non-demented controls ($^{**}p < 0.01$, Mann-Whitney U test). **b** A receiver-operating characteristic (ROC) curve of the detection of the plasma CNAs in the AD patients. The area under the ROC curve was 0.807 for distinguishing AD patients from non-demented control individuals

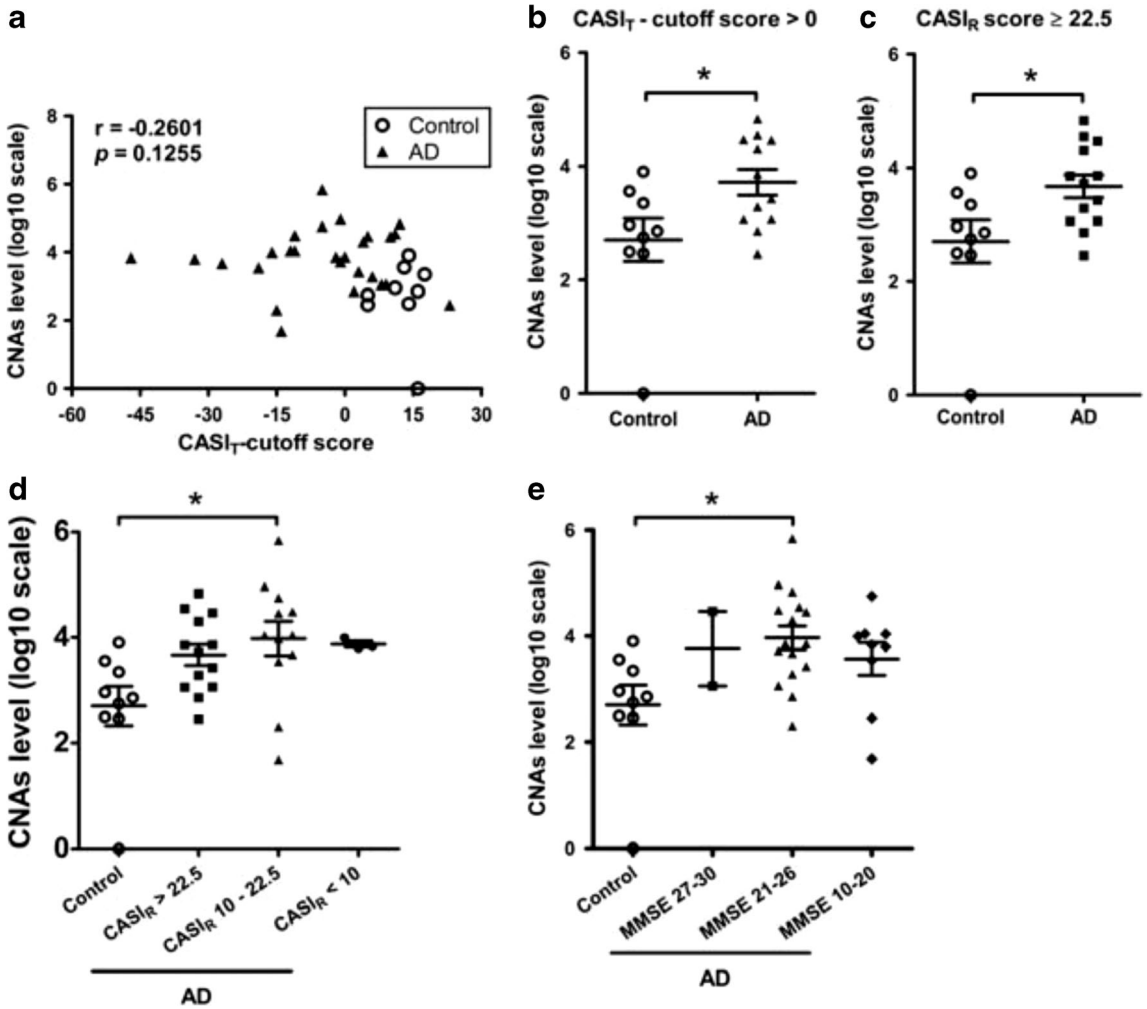


Fig. 3 The plasma levels of CNA are increased in subjects with AD before the onset of cognitive dysfunction. **a** Spearman's correlation analysis between the CNA levels (shown as a $\log_{10}$ scale) and cognitive function [defined as the Cognitive Ability Screening Instrument total ($CASI_T$)-cutoff score] in all subjects. The circles represent the non-demented controls, and the filled triangles represent the AD patients. The plasma levels of CNA in the AD patients and normal cognition were higher than those of the non-demented controls despite the $CASI_T$-cutoff score (**b**) or CASI of the combined short-term memory and orientation domain scores ($CASI_R$) score (**c**). **d**, **e** Comparison of the CNA levels between the non-demented controls and AD patients who were stratified according to their cognitive function test scores. Note that increased CNA levels were observed in the AD patients who had mild cognitive impairment (MCI). The data are expressed as mean ± SEM. $*p < 0.05$, Mann-Whitney *U* test in **b** and **c**; Kruskal-Wallis test in **d** and **e**

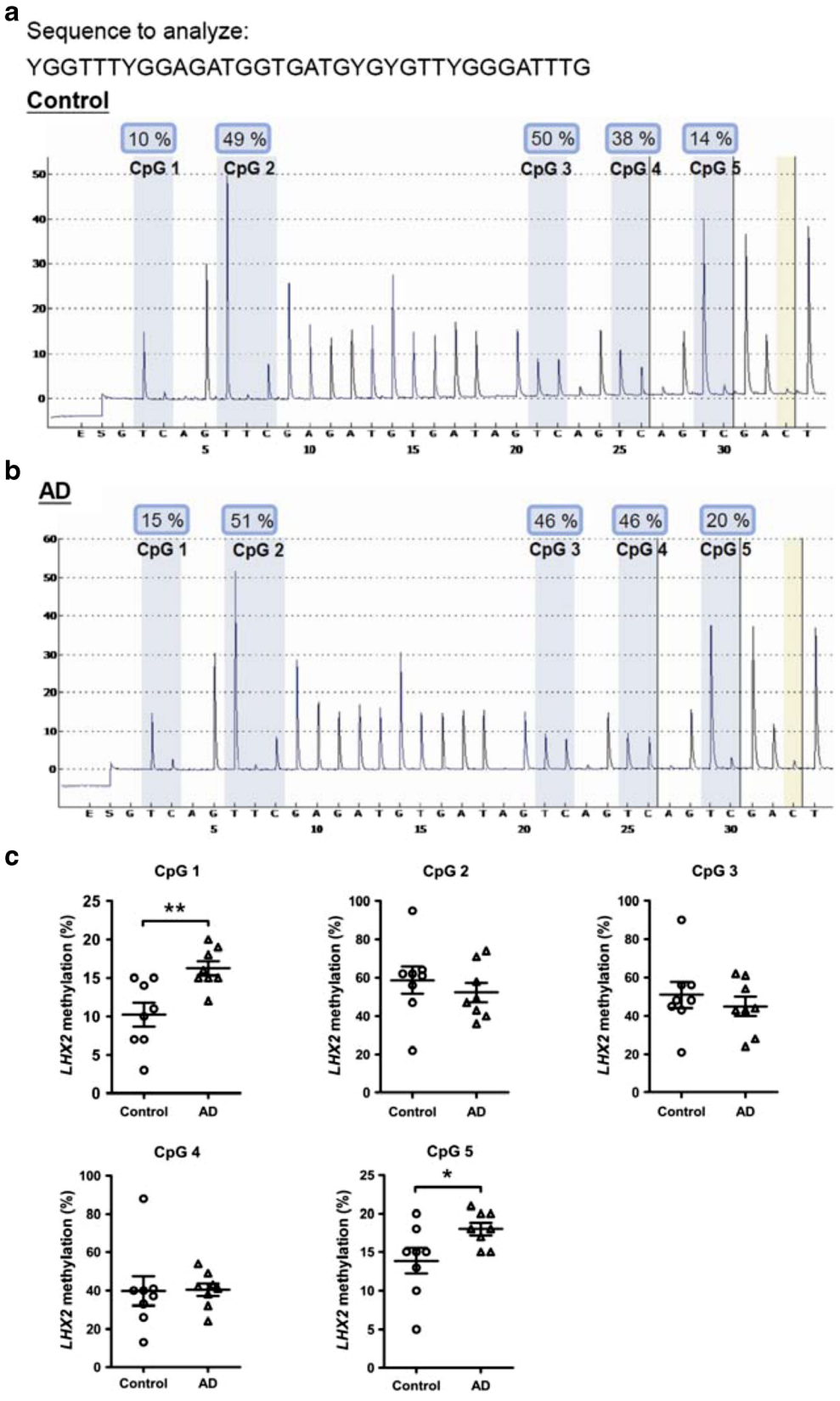
Table 1 Spearman's correlation analysis of the plasma levels of CNA and cognitive function in patients with Alzheimer's disease (AD)

Patients with AD	Plasma CNA levels	
	Correlation coefficient	*p* value
$CASI_T$-cutoff		
Difference > 0 (normal)	0.1538	0.6331
Difference < 0 (dysfunction)	0.526	0.044
$CASI_R$ score		
Score ≥ 22.5 (normal)	0.2330	0.4436
Score < 22.5 (dysfunction)	0.5573	0.0384

$CASI_T$ Cognitive Ability Screening Instrument-total, *$CASI_R$* Cognitive Ability Screening Instrument-combined short-term memory and orientation domain scores

cognition (score, 27–30), mild cognitive impairment (MCI; score, 21–26), or moderate cognitive impairment (score, 10–20), as previously described [20]. Increasing CNA concentrations were observed in patients with AD who were categorized with MCI (Fig. 3e; $*p < 0.05$).

Fig. 4 Highly methylated neuronal tissue-specific *LHX2* was found in ▶ plasma CNAs of AD patients. **a**, **b** A pyrosequencing analysis shows the pattern of *LHX2* methylation in non-demented controls (**a**) and AD patients (**b**). The percentage of methylation in each CpG dinucleotide was calculated using analysis software. There were five CpG sites (CpG sites 1–5) in the analyzed sequence. **c** The levels of *LHX2* methylation in CpG sites 1 and 5 in the AD patients were significantly higher than those in the controls. The data are expressed as mean ± SEM. $*p < 0.05$, Mann-Whitney *U* test


a
Sequence to analyze:
YGGTTTYGGAGATGGTGATGYGYGTTYGGGATTTG
Control
10 %
CpG 1
49 %
CpG 2
50 %
CpG 3
38 %
CpG 4
14 %
CpG 5
b
AD
15 %
CpG 1
51 %
CpG 2
46 %
CpG 3
46 %
CpG 4
20 %
CpG 5
c
CpG 1
CpG 2
CpG 3
CpG 4
CpG 5
LHX2 methylation (%)
Control
AD
**
*

Highly Methylated Neuronal Tissue-Specific *LHX2* Gene Was Found in the Plasma CNAs of Patients with AD

To investigate whether the increased CNA concentrations observed in patients with AD resulted from neuronal tissue destruction, we examined the methylation profile of *LHX2* in the CNAs (Fig. 4a, b). We selected six individuals from each group and used a pyrosequencing technique to examine the 192-bp sequence of *LHX2*, which contains 10 CpG sites. We found that CpG sites 1 and 5 of *LHX2* were highly methylated in CNAs of patients with AD (Fig. 4c). These results suggested that the increased plasma CNA levels in patients with AD could be attributed to the destruction of brain cells.

Discussion

The results of the present study demonstrated that aggregated Aβ-induced cell death correlated with increased plasma CNA levels, before the formation of Aβ plaques due to Aβ accumulation. The pyrosequencing data demonstrated that the percentage of methylated *LHX2* in plasma CNAs from patients with AD was higher than that from non-demented controls, inferring that the upregulation of the loss of neuronal cells enhanced the production of CNAs through cell apoptosis in patients with AD. The results from the present study outline potential for developing a new diagnostic method for early disease progression of AD.

A previous study demonstrated that 10 μM of $A\beta_{1-42}$ treatment induced apoptosis in SH-SY5Y cells [30] and apoptotic cells, but not necrotic cells, and released significantly more DNA into the media compared with untreated cells [31]. We also demonstrated that Aβ promoted the apoptosis of SH-SY5Y cells (Fig. 1a). Further, CNA levels in the culture medium of SH-SY5Y cells were increased after Aβ stimulation. Notably, we found that the plasma CNA levels were significantly increased in Tg2576 mice, with levels peaking at 6–8 months (Fig. 1c). Previous studies reported that Tg2576 mice developed memory deficits in middle age (6–14 months), with deficits caused by the extracellular accumulation of soluble Aβ in the absence of amyloidosis [32]. In addition, other in vitro studies reported that Aβ oligomers are more toxic to cortical neurons than fibrillary Aβ. Oligomers induce apoptosis through a phospholipase C-mediated mechanism that involves Ca^{2+} release from the endoplasmic reticulum [33]. Aβ-induced apoptosis was abolished by pre-treatment with antibodies against Aβ oligomers [34]. Thus, these findings demonstrated that Aβ oligomers could arguably account for the primary cause of neuronal cell death in AD. Therefore, our findings in Tg2576 mice implied that higher levels of plasma CNAs may represent the formation of Aβ oligomers and Aβ oligomer-induced neuronal death in the brain. However, we also found that CNAs were not increased in parallel to the deterioration of cognitive dysfunction (Fig. 1d; Fig. 3d, e). Previous studies reported that the half-life of CNAs in circulation ranges between 15 min to several hours [4, 35]. Moreover, we can infer that plasma CNAs of Tg2576 mice in the peak months reflect the beginning of neuronal cell death and middle level of impaired cognitive function. Thus, regardless of the cognitive level of patients with AD, the mean CNA concentration in AD populations was higher than those of non-demented controls, especially in populations with MCI or mild/moderate AD.

To address the question of how CNAs from the brain cross the blood-brain barrier (BBB), a recent study identified that DNAs within glioma-derived extracellular vesicles crossed intact BBB and were able to be isolated from peripheral blood [36]. This is perhaps the only evidence to date to address the question about how cell-free DNAs cross the BBB by practical experiments without causing any brain damage. Another study in our previous discussion also demonstrated the concentration of methylated cell-free plasma DNAs in patients with relapsing-remitting multiple sclerosis (MS) was higher than that of the healthy control [9]. MS is a chronic inflammatory disease of the CNS combined with oligodendrocyte and neuronal injury. Just like MS, AD belongs to neurodegenerative diseases characterized by Aβ-induced synaptic dysfunction, chronic neuroinflammation, and neuronal death. Although the cellular origins of CNAs were not clearly identified, these evidences have given us a hint that pathological neuronal death may cause elevated CNAs to be released from apoptotic cells to the plasma through the BBB.

The mechanisms underlying the increases in the CNAs in the plasma of patients with AD are still unclear. According to accumulating evidence, apoptosis plays a major role in the generation of CNAs [37–39]. This evidence is strengthened by the size of the CNAs, which are primarily 180–200 bp, which is in accordance with the characteristics of the oligonucleosomal ladder in apoptotic cell death. In contrast, DNA fragments larger than 10,000 bp are predominant in cells undergoing necrosis [38]. Therefore, we can infer that the upregulation of the loss of neuronal cells enhances the production of CNAs through cell apoptosis in patients with AD.

We also used the levels of β-globin as a content marker of CNAs. However, β-globin is found in all nuclear cell bodies. Therefore, we cannot exclude the possibility that other pathologies associated with cell death or DNA release contributed to increased plasma concentration of CNAs. To decrease the effects of any confounding factors in the experiment, we excluded cases with comorbidities (Supplemental Table S1). The AUC of 0.807 indicated excellent diagnostic accuracy.

Methylation analyses of CNAs can now be used as a tool for the detection of early cancer [26, 40, 41]. Methylation plays an important role in carcinogenesis and tissue-specific identity [42, 43]. Notably, *LHX2* is highly methylated in

neuronal tissues compared to non-neuronal tissues [15]. Detection of the methylation profile in *LHX2* may indicate the occurrence of neural tissue destruction and is suitable for application in the diagnosis of brain disorders. The results of our pyrosequencing analysis showed that the percentage of *LHX2* methylation in the plasma CNAs, particularly on CpG sites 1 and 5, was higher in patients with AD than the percentage in non-demented controls (Fig. 4). Therefore, measuring the levels of neuronal-specific methylation of CNAs may provide a new approach for the diagnosis of early AD progression.

Conclusion

Our data demonstrated that the increased plasma levels of CNAs were associated with cell death that was induced by Aβ toxicity in models of AD, in vitro and in vivo, and in patients, particularly at the early stage of the disease. Additionally, we identified different levels of *LHX2* methylation between non-demented controls and patients with AD. Taken together, the increased levels of plasma CNAs and higher methylation status of *LHX2* can serve as novel markers used for the detection of neuronal damage in patients with AD.

Acknowledgements The authors thank M.-T. Yu, C.-H. Pan, and Y.-T. Sun for their technical assistance in the study.

Abbreviations and Acronyms *CNAs*, circulating nucleic acids; AD Alzheimer's disease; *LHX2*, LIM homeobox 2 gene *aCSF*, artificial cerebrospinal fluid; *CDR*, Clinical Dementia Rating; *MMSE*, Mini-Mental State Examination; *CASI*, Cognitive Abilities Screening Instrument; *MS*, multiple sclerosis

Author Contributions K.-J. Tsai, Y.-M. Kuo, and M.-C. Pai contributed substantially to the conception and design of the research project and its scientific interpretation; K.-J. Tsai wrote the manuscript; I.-F. Wang and P.-M. Chiang took responsibility for the statistical analysis and reporting of the results.

Funding Information This study was supported by grants from the NCKU Hospital Medical Center (NCKUH-10006002); Ministry of Health, Taiwan (DOH101-TD-B-111-0002); and Ministry of Science and Technology, Taiwan (MOST-104-2745-B-006-001, MOST-105-2628-B-006-016-MY3, and MOST-106-2628-B-006-001-MY4).

Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no competing interests.

References

1. Lu PH, Edland SD, Teng E, Tingus K, Petersen RC, Cummings JL, Alzheimer's Disease Cooperative Study Group (2009) Donepezil delays progression to AD in MCI subjects with depressive symptoms. Neurology 72(24):2115–2121. https://doi.org/10.1212/WNL.0b013e3181aa52d3
2. Galimberti D, Scarpini E (2011) Disease-modifying treatments for Alzheimer's disease. Ther Adv Neurol Disord 4(4):203–216. https://doi.org/10.1177/1756285611404470
3. Traver S, Assou S, Scalici E, Haouzi D, Al-Edani T, Belloc S, Hamamah S (2014) Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy. Hum Reprod Update 20(6):905–923. https://doi.org/10.1093/humupd/dmu031
4. Schwarzenbach H (2013) Circulating nucleic acids as biomarkers in breast cancer. Breast Cancer Res : BCR 15(5):211. https://doi.org/10.1186/bcr3446
5. Chang CP, Chia RH, Wu TL, Tsao KC, Sun CF, Wu JT (2003) Elevated cell-free serum DNA detected in patients with myocardial infarction. Clinica Chimica Acta; Int J Clinical Chemistry 327(1–2):95–101
6. Rainer TH, Wong LK, Lam W, Yuen E, Lam NY, Metreweli C, Lo YM (2003) Prognostic use of circulating plasma nucleic acid concentrations in patients with acute stroke. Clin Chem 49(4):562–569
7. Lo YM, Rainer TH, Chan LY, Hjelm NM, Cocks RA (2000) Plasma DNA as a prognostic marker in trauma patients. Clin Chem 46(3):319–323
8. Fox A, Gal S, Fisher N, Smythe J, Wainscoat J, Tyler MP, Watt SM, Harris AL (2008) Quantification of circulating cell-free plasma DNA and endothelial gene RNA in patients with burns and relation to acute thermal injury. Burns : J Int Soc Burn Injuries 34(6):809–816. https://doi.org/10.1016/j.burns.2007.10.003
9. Liggett T, Melnikov A, Tilwalli S, Yi Q, Chen H, Replogle C, Feng X, Reder A et al (2010) Methylation patterns of cell-free plasma DNA in relapsing-remitting multiple sclerosis. J Neurol Sci 290(1–2):16–21. https://doi.org/10.1016/j.jns.2009.12.018
10. Sandberg R, Yasuda R, Pankratz DG, Carter TA, Del Rio JA, Wodicka L, Mayford M, Lockhart DJ et al (2000) Regional and strain-specific gene expression mapping in the adult mouse brain. Proc Natl Acad Sci U S A 97(20):11038–11043
11. Letwin NE, Kafkafi N, Benjamini Y, Mayo C, Frank BC, Luu T, Lee NH, Elmer GI (2006) Combined application of behavior genetics and microarray analysis to identify regional expression themes and gene-behavior associations. J Neurosci 26(20):5277–5287. https://doi.org/10.1523/JNEUROSCI.4602-05.2006
12. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS et al (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445(7124):168–176. https://doi.org/10.1038/nature05453
13. Roth RB, Hevezi P, Lee J, Willhite D, Lechner SM, Foster AC, Zlotnik A (2006) Gene expression analyses reveal molecular relationships among 20 regions of the human CNS. Neurogenetics 7(2):67–80. https://doi.org/10.1007/s10048-006-0032-6
14. Ladd-Acosta C, Pevsner J, Sabunciyan S, Yolken RH, Webster MJ, Dinkins T, Callinan PA, Fan JB et al (2007) DNA methylation signatures within the human brain. Am J Hum Genet 81(6):1304–1315. https://doi.org/10.1086/524110
15. Ghosh S, Yates AJ, Fruhwald MC, Miecznikowski JC, Plass C, Smiraglia D (2010) Tissue specific DNA methylation of CpG islands in normal human adult somatic tissues distinguishes neural from non-neural tissues. Epigenetics 5(6):527–538
16. Lichtenstein AV, Melkonyan HS, Tomei LD, Umansky SR (2001) Circulating nucleic acids and apoptosis. Ann N Y Acad Sci 945:239–249
17. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P (2001) About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. Clin Chim Acta 313(1–2):139–142
18. Tsai KJ, Tsai YC, Shen CK (2007) G-CSF rescues the memory impairment of animal models of Alzheimer's disease. J Exp Med 204(6):1273–1280. https://doi.org/10.1084/jem.20062481

19. Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1(5):345–347. https://doi.org/10.1038/ng0892-345
20. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 34(7):939–944
21. Morris JC (1993) The clinical dementia rating (CDR): current version and scoring rules. Neurology 43(11):2412–2414
22. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ et al (1998) Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 62(4):768–775. https://doi.org/10.1086/301800
23. Folstein MF, Folstein SE, McHugh PR (1975) "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 12(3):189–198
24. Teng EL, Hasegawa K, Homma A, Imai Y, Larson E, Graves A, Sugimoto K, Yamaguchi T et al (1994) The Cognitive Abilities Screening Instrument (CASI): a practical test for cross-cultural epidemiological studies of dementia. Int Psychogeriatrics / IPA 6(1): 45–58 **discussion 62**
25. Tsai RC, Lin KN, Wu KY, Liu HC (2004) Improving the screening power of the cognitive abilities screening instrument, Chinese version. Dement Geriatr Cogn Disord 18(3–4):314–320. https://doi.org/10.1159/000080125
26. Vaissiere T, Cuenin C, Paliwal A, Vineis P, Hoek G, Krzyzanowski M, Airoldi L, Dunning A et al (2009) Quantitative analysis of DNA methylation after whole bisulfitome amplification of a minute amount of DNA from body fluids. Epigenetics 4(4):221–230
27. Butt AN, Swaminathan R (2008) Overview of circulating nucleic acids in plasma/serum. Ann N Y Acad Sci 1137:236–242. https://doi.org/10.1196/annals.1448.002
28. Urbanova M, Plzak J, Strnad H, Betka J (2010) Circulating nucleic acids as a new diagnostic tool. Cell Mol Biol Lett 15(2):242–259. https://doi.org/10.2478/s11658-010-0004-6
29. Zhu S, Stavrovskaya IG, Drozda M, Kim BY, Ona V, Li M, Sarang S, Liu AS et al (2002) Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. Nature 417(6884):74–78. https://doi.org/10.1038/417074a
30. Qin ZX, Zhu HY, Hu YH (2009) Effects of lysophosphatidylcholine on beta-amyloid-induced neuronal apoptosis. Acta Pharmacol Sin 30(4):388–395. https://doi.org/10.1038/aps.2009.25
31. Choi JJ, Reich CF 3rd, Pisetsky DS (2004) Release of DNA from dead and dying lymphocyte and monocyte cell lines in vitro. Scand J Immunol 60(1–2):159–166. https://doi.org/10.1111/j.0300-9475.2004.01470.x
32. Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. Nature 440(7082):352–357. https://doi.org/10.1038/nature04533
33. Resende R, Ferreiro E, Pereira C, Resende de Oliveira C (2008) Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. Neuroscience 155(3):725–737. https://doi.org/10.1016/j.neuroscience.2008.06.036
34. Reese LC, Zhang W, Dineley KT, Kayed R, Taglialatela G (2008) Selective induction of calcineurin activity and signaling by oligomeric amyloid beta. Aging Cell 7(6):824–835. https://doi.org/10.1111/j.1474-9726.2008.00434.x
35. Botezatu I, Serdyuk O, Potapova G, Shelepov V, Alechina R, Molyaka Y, Ananev V, Bazin I et al (2000) Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. Clin Chem 46(8 Pt 1):1078–1084
36. Garcia-Romero N, Carrion-Navarro J, Esteban-Rubio S, Lazaro-Ibanez E, Peris-Celda M, Alonso MM, Guzman-De-Villoria J, Fernandez-Carballal C et al (2017) DNA sequences within glioma-derived extracellular vesicles can cross the intact blood-brain barrier and be detected in peripheral blood of patients. Oncotarget 8(1):1416–1428. https://doi.org/10.18632/oncotarget.13635
37. Giacona MB, Ruben GC, Iczkowski KA, Roos TB, Porter DM, Sorenson GD (1998) Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas 17(1):89–97
38. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R (2001) DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 61(4):1659–1665
39. Suzuki N, Kamataki A, Yamaki J, Homma Y (2008) Characterization of circulating DNA in healthy human plasma. Clinica Chimica Acta; Int J Clinical Chemistry 387(1–2):55–58. https://doi.org/10.1016/j.cca.2007.09.001
40. Rykova EY, Tsvetovskaya GA, Sergeeva GI, Vlassov VV, Laktionov PP (2008) Methylation-based analysis of circulating DNA for breast tumor screening. Ann N Y Acad Sci 1137:232–235. https://doi.org/10.1196/annals.1448.021
41. Li M, Chen WD, Papadopoulos N, Goodman SN, Bjerregaard NC, Laurberg S, Levin B, Juhl H et al (2009) Sensitive digital quantification of DNA methylation in clinical samples. Nat Biotechnol 27(9):858–863. https://doi.org/10.1038/nbt.1559
42. Schilling E, Rehli M (2007) Global, comparative analysis of tissue-specific promoter CpG methylation. Genomics 90(3):314–323. https://doi.org/10.1016/j.ygeno.2007.04.011
43. Illingworth R, Kerr A, Desousa D, Jorgensen H, Ellis P, Stalker J, Jackson D, Clee C et al (2008) A novel CpG island set identifies tissue-specific methylation at developmental gene loci. PLoS Biol 6(1):e22. https://doi.org/10.1371/journal.pbio.0060022